Effective Use of PI3K and MEK Inhibitors to Treat Mutant K-Ras G12D and PIK3CA H1047R Murine Lung Cancers

Jeffrey A. Engelman[1,2,3*], Liang Chen[4,5*], Xiaohong Tan[4,5], Katherine Crosby[6], Alexander Guimaraes[7], Rabi Upadhyay[7], Michel Maira[8], Kate McNamara[4,5], Samanthi A. Perera[4,5], Youngchul Song[1], Lucian R. Chirieac[9], Ramneet Kaur[1], Angela Lightbown[6], Jessica Simendinger[6], Timothy Li[2], Robert F. Padera[9], Carlos García-Echeverría[8], Ralph Weissleder[7], Umar Mahmood[7], Lewis C. Cantley[2,3,10#], Kwok-Kin Wong[3,4,5#]

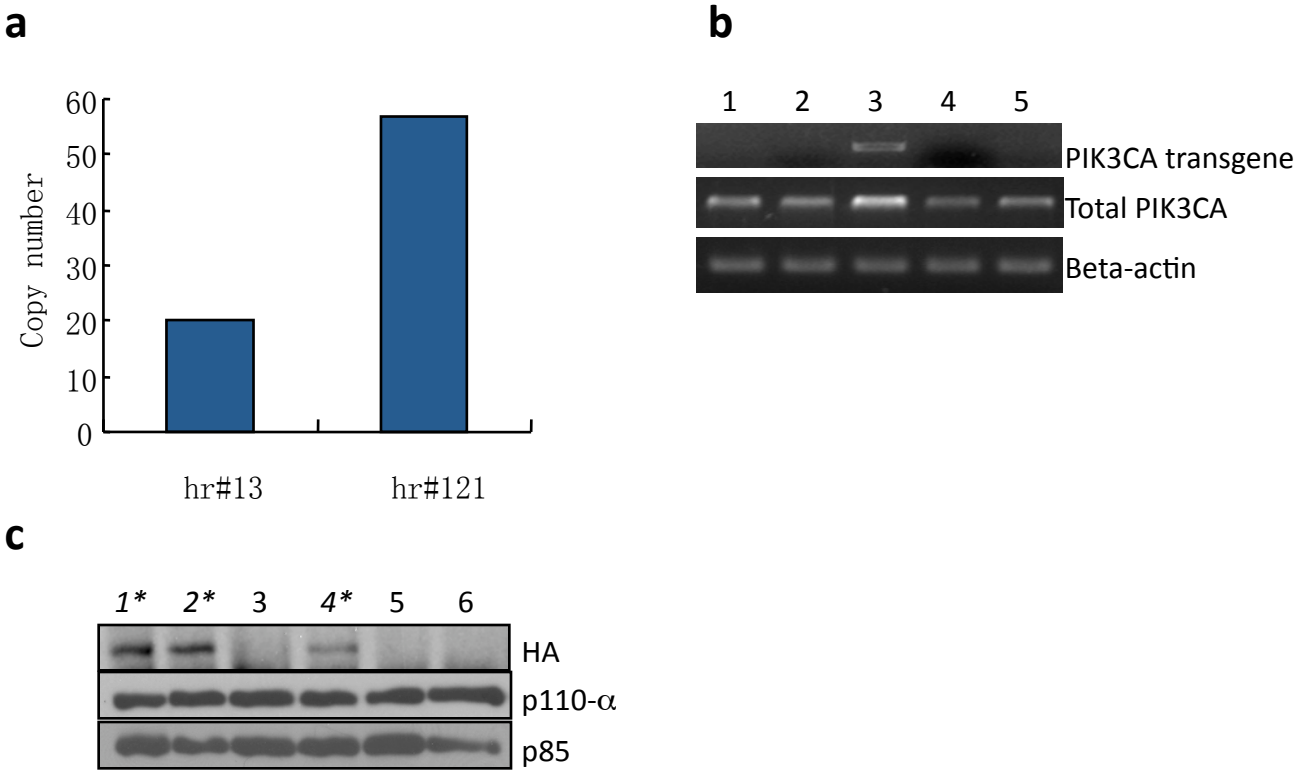
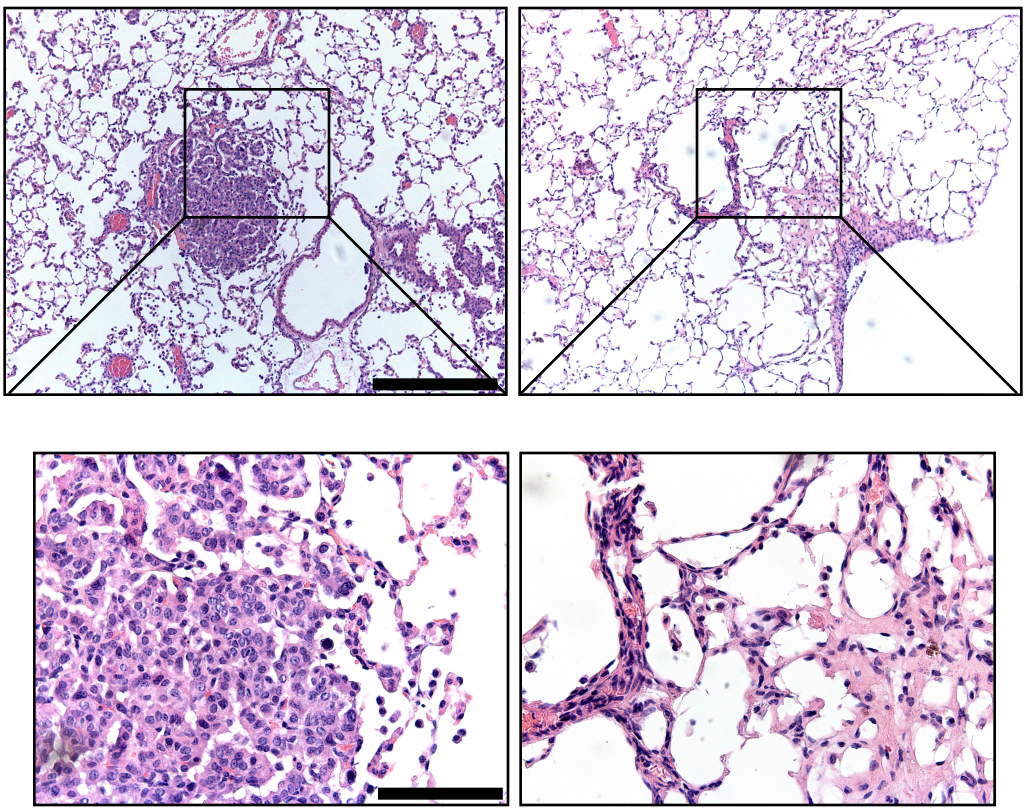


Figure S1. PIK3CA H1047R is expressed in a doxycycline inducible manner. (**a**) The copy number of the *Tet-op PIK3CA H1047R* was determined by quantitative PCR using primers specific for the transgene. The copy numbers for founder lines #13 and #121 are shown. (**b**) Levels of PIK3CA H1047R transcript were evaluated by RT-PCR. RNA was harvested from *Tet-op PIK3CA H1047R /CCSP-rtTA* mice that had not been on doxy (lane 1), and from those that had received doxy for 6 weeks (lane 2), 12 weeks (lane 3), 12 weeks followed by 1 week of doxy withdrawal (lane 4), and 12 weeks followed by 3 weeks of doxy withdrawal (lane 5). Primers used to amplify total PIK3CA and β-actin were used as controls. (**c**) Expression of the mutant p110-α H1047R ((with a C-terminal HA tag [2])) protein in the bitransgenic *Tet-op PIK3CA H1047R /CCSP-rtTA* mice induced with doxycycline. Protein extracts were made from the lungs of the *Tet-op PIK3CA H1047R /CCSP-rtTA* mice and were immunooprecipitated with an anti-p85 antibody. The immunoprecipitates were probed with the indicated antibodies. Mice 1,2, and 4 were on doxy for 12 weeks while 3,5, and 6 were sibling controls that were not put on a doxycycline diet.

Figure S1

Second H1047R founder with Doxy induced tumors



9 months on doxy

7 months on doxy, 3 weeks off doxy

Figure S2. Tumors induced by *Tet-op PIK3CA H1047R* in founder line #121 require continuous doxycycline. *Left)* Histological analysis of lungs after 9 months of doxy induction revealed adenocarcinomas. *Right)* After 3 weeks of doxycycline withdrawl, there was marked tumor shrinkage by MRI (data not shown) and no evidence of tumors by histological analysis. Scale is 200μM and 50μM for upper and lower panels respectively.

Figure S2

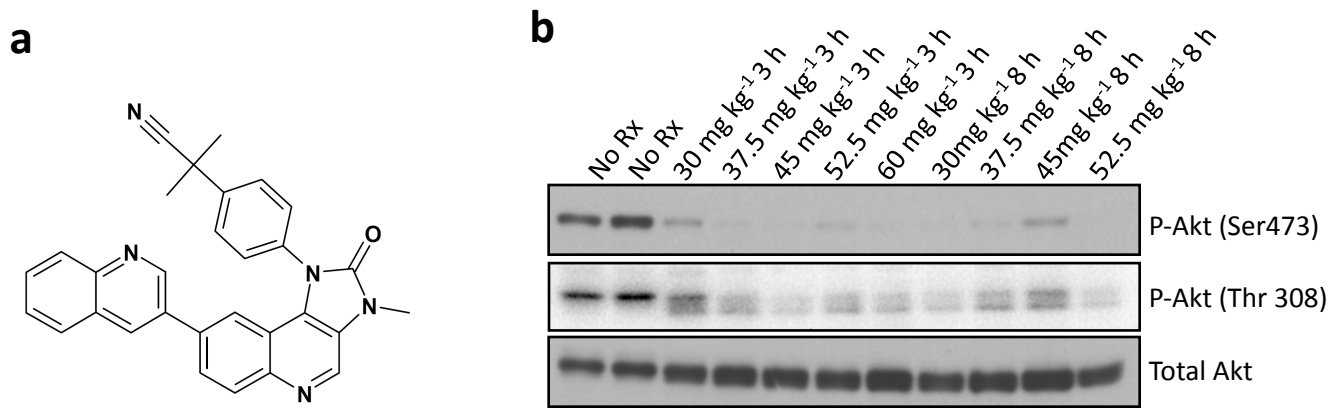


Figure S3. NVP-BEZ235 inhibits PI3K signaling in mouse lungs. (**a***)* Chemical structure of NVP-BEZ235. (**b**) Control mice were administered one of the indicated doses of NVP-BEZ235, and lungs were harvested either 3 or 8 hours later. Protein lysates from those lungs were probed with antibodies against P-Akt (Ser473), P-Akt (Thr308) and total Akt.

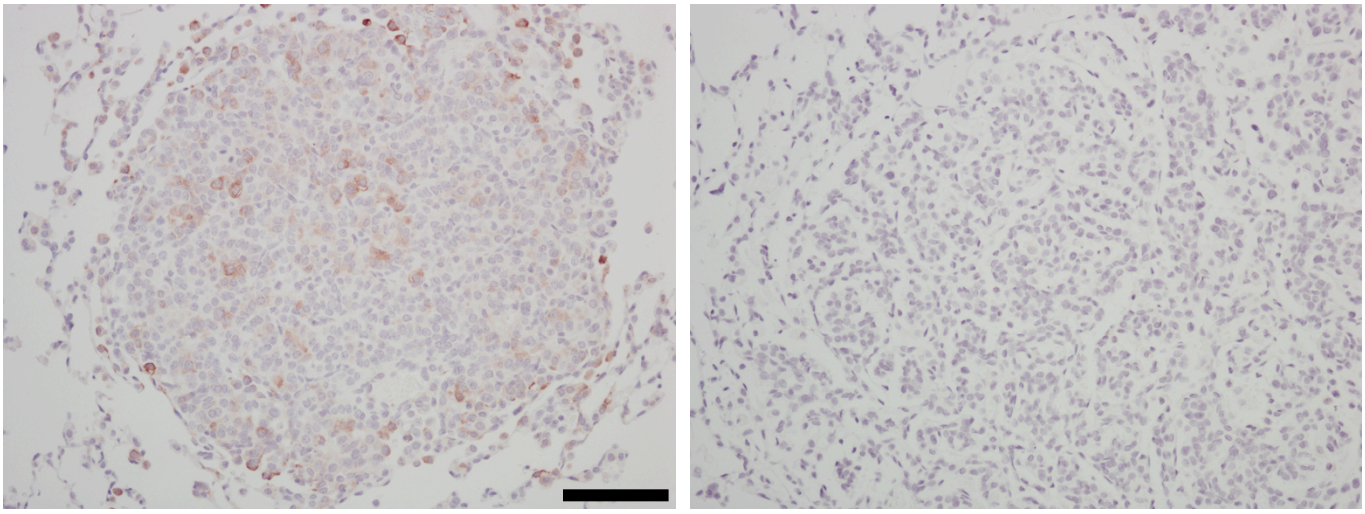
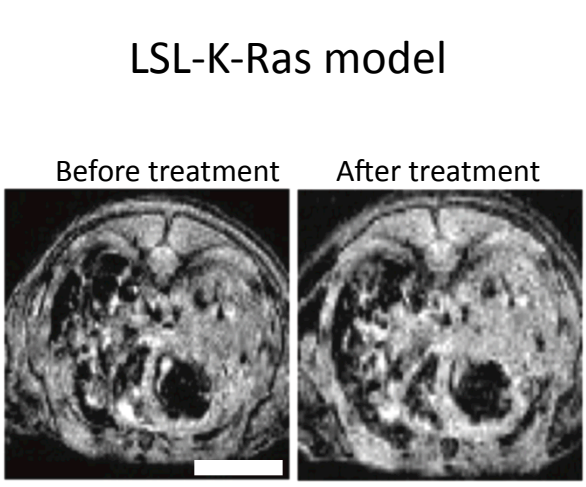


Figure S4. Rapamycin leads to loss of P-S6 levels in p110-α H1047R induced lung tumors. *Tet-op PIK3CA H1047R /CCSP-rtTA* mice were induced to develop tumors with doxycycline. After tumors developed, mice were treated with either placebo or rapamycin 6 mg/kg daily for two weeks. Upon completion of treatment, lungs were harvested. P-S6 levels were determined by IHC. Scale is 100μM.

a



b

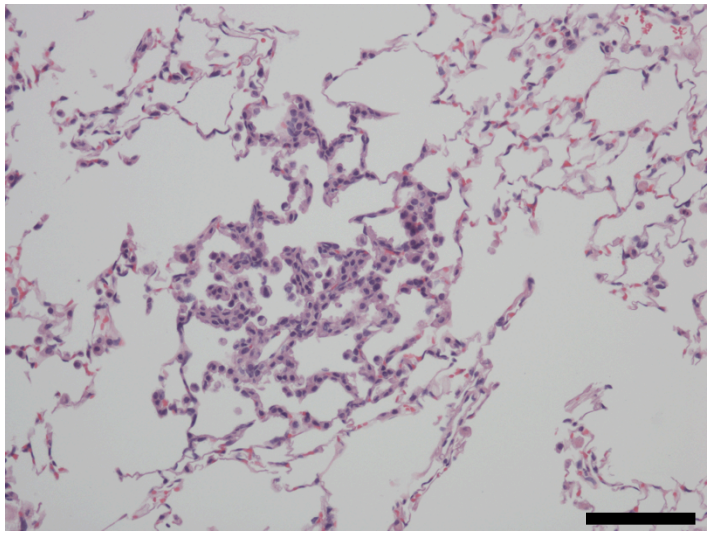


Figure S5. LSL K-Ras induced tumors do not respond to single-agent PI3K inhibitors but are effectively treated by a combination of PI3K and MEK inhibitors. ***(a)*** *LSL-K-Ras* mice were treated with AdenoCre and followed until tumors developed as documented by MRI. The tumors were treated with NVP-BEZ235 35mg/kg per day for two weeks. Axial MR images taken before and after treatment of a representative mouse is shown. Please note that there is no significant shrinkage of the tumors. Scale is 4.5 mm. (**b**) *LSL-K-Ras* were induced to develop lung tumors and treated with the various treatment regimens describe elsewhere (**Fig. 4**). Lungs treated with NVP-BEZ235 + ARRY-142886 for 2 weeks had very little residual tumor by pathological examination. Shown is a hematoxylin and eosin stain demonstrating one of the more prominent tumor remnants that we observed. Scale is 200μM. MRI is displayed elsewhere (**Fig. 4a**).

Figure S5

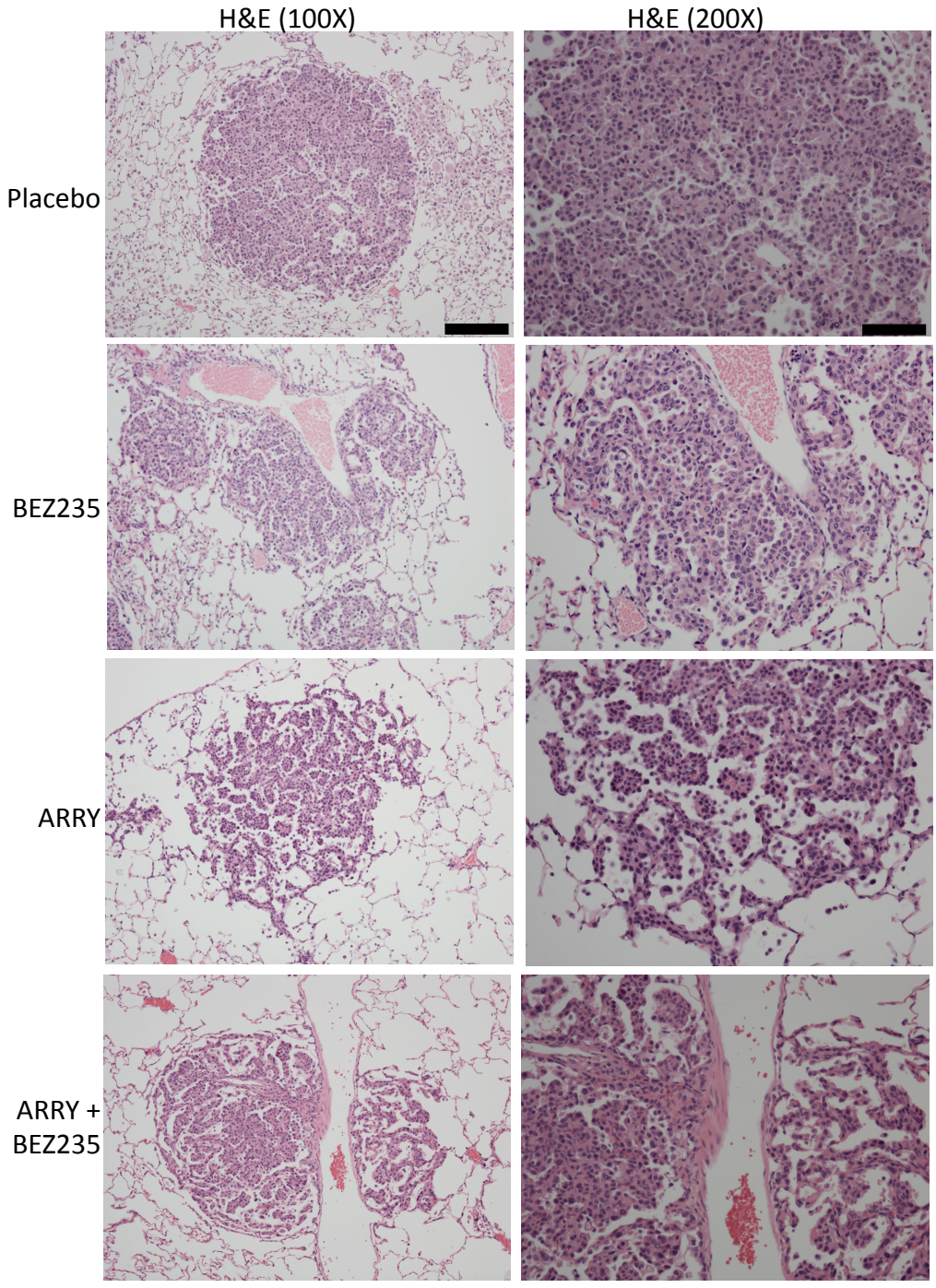


Figure S6. Hematoxylin and eosin stains of tumor nodules that were assessed by immunohistochemistry in the main text (Fig. 4d). Scale is 200μM and 100μM for the left and right panels respectively.

Figure S6

Supplementary Materials and Methods

Immunohistochemical analysis

Slides were deparaffinized in three changes of xylene and rehydrated through graded ethanols. Antigen retrieval was performed using 10mM citrate buffer, pH 6.0. Slides were quenched in 3% hydrogen peroxide and blocked with TBST/5% normal goat serum. Primary antibodies were diluted in TBST/5% normal goat serum (P-S6 Ribosomal Protein (Ser235/236) (D57.2.2E) Rabbit mAb (CST #4858), P-p44/42 MAPK (Erk1/2) (Thr202/ Tyr204) (D13.14.4E) Rabbit mAb (CST#4370) and P-4E-BP1 (Thr37/46) (236B4) Rabbit mAb (CST #2855)) or SignalStain® Antibody Diluent (CST #8112) (P-Akt (Ser473) (D9E) Rabbit mAb (CST #4060) and incubated overnight at 4 degrees. Detection was performed using Vector ABC Elite (Vector Laboratories) and NovaRed (Vector Laboratories).

Immunoblotting

Lungs were removed from the mice and snap-frozen in liquid nitrogen. They lungs were then homogenized in 1% NP-40 lysis buffer (20 mM Tris, pH 7.4/150 mM NaCl/1% Nonidet P-40/ 10% glycerol/1 mM EDTA/1 mM EGTA/5 mM sodium pyrophosphate/50 mM NaF/10 nM b -glycerophosphate/1 mM sodium vanadate/0.5 mM DTT/4 μg/ml leupeptin/4 μg/ml pepstatin/4 μg/ml apoprotein/1 mM PMSF). P-AKT (Ser473), P-AKT (Thr308), P-MAPK (P-Erk1/2) (Thr202/Tyr204), and total MAPK (Erk1/2) antibodies were purchased from Cell Signaling Technology. Total AKT antibody was purchased from Santa Cruz Biotechnology.

PCR analysis

To quantify the mRNA expression level of PI3KCA H1047R, primers covering HA Tag were designed, along with upstream sequence. Antisense (lc231 annealing to HA tag): 5'-GCATAGTCAGGCACGTCGTA-3' and sense (lc232): 5'-CGTGTGCCATTTGTTTTGAC-3'. To quantify total PI3KCA expression in the mouse, primers were designed in the region that are identical in transgene (human PI3KCA) and endogenous (mouse PI3KCA). Sense (lc257): 5'-AAAAAGGCCACTGTGGTTGAAT-3' and antisense (lc256):5'-ACAGGTCAATGGCTGCATCATA-3'.